Minireview

Gene-encoded peptide antibiotics and innate immunity.

Do 'animalcules' have defence budgets?

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Abstract Gene-encoded peptide antibiotics have been isolated from plants, animals and microbes. Their protective role has been related to innate immunity, which has gradually become accepted across the biomedical community. The evidence for the immune function of peptide antibiotics has been convincingly demonstrated by a combination of both in vitro and in vivo data for plants and insects, but for vertebrates in vivo data are scarce. Using frogs as model systems, it has been shown that the genes for antibacterial peptides are down-regulated by glucocorticoids, while IκBα is clearly up-regulated. Experimental infections with frog bacteria have shown that the normal capacity to control the natural flora is lost after treatment with glucocorticoids. A low-specificity immune mechanism is cost-effective, something that may have been of importance during animal evolution.

Key words: Peptide antibiotic; Innate immunity; NFκB-IκB; Glucocorticoid; Amphibian; *Rana esculenta*

1. Introduction

Gene-encoded peptide antibiotics have finally come of age. The number of such peptides identified has rapidly grown to several hundreds in the last years, being isolated from plants, animals and microbes (for reviews, see [1–4]). They are known to protect the host against infections and this role was first demonstrated in insects [5]. In plants this protection has been exploited through genetic engineering to prevent fungal or bacterial infections of crops [2]. When it comes to vertebrates and mammals, quite a number of peptides have been isolated and proven to be highly effective in vitro, but currently in vivo data are scarce.

The protective role of peptide antibiotics has been related to immunity and the concept of 'innate immunity' has gradually become accepted throughout the biomedical community. The evidence for the immune function of peptide antibiotics has been convincingly demonstrated by both in vitro and in vivo experiments in insects [6,7]. In the case of the first animal peptides isolated in the beginning of the 1980s [8,9], their role in immunity was clearly identified. In humans, this demonstration was recently provided by the finding that terminal infections of airways with *Pseudomonas aeruginosa* in patients with cystic fibrosis is due to salt inactivation of the human β-defensin 1 [10].

2. The superfamilies of peptide antibiotics

Chemically speaking peptide antibiotics can be divided in three main classes, one may say superfamilies, because the sequence similarities within each group are almost negligible. Rather it is the gross composition and the 3D structure that form the basis for their grouping, as illustrated in Fig. 1.

2.1. Group I: linear, α-helical peptides without cysteines

This family includes the cecropins [1], the magainin/PGLa-like peptides [11,12], the bombinins [13–15] and the smaller temporins [16]. The sequences of temporin B and the bombinin-like peptide, BLP-3, as described by Gibson et al. [14], are given in Fig. 1 and their antibacterial activity in Table 1.

2.2. Group II: peptides with an even number of cysteines intralinked by disulfide bridges

The number of such bonds can vary from one to four. Peptides with one disulfide forming a C-terminal loop are chiefly found in frog skin secretions (brevinins, esculentins [17]). The sequences of brevinin 2E [18] and brevinin 2T from *Rana temporaria* (unpublished), as well as their activities, are reported in Fig. 1 and Table 1, respectively. Examples of peptides with two disulfide bridges are the tachyplesins from horseshoe crab [19] and the protegrins from pig [4]. The best studied within the Cys-containing group are the mammalian defensins with three S–S bonds. Interestingly, in α- and β-defensins two different disulfide arrangements (see Fig. 1) can give almost the same antiparallel β-sheet structure. These molecules have been very well studied in a number of mammals, including humans [4]. The α-defensins are synthesized during the maturation of neutrophils and then stored in special granules, while the β-defensins are made in the epithelia of the airways and the intestine. Insects and plants also have defensins, in these cases comprising a short α-helix packed on one side of the antiparallel β-sheet structure.

2.3. Group III: peptides with an unusually high proportion of one or two amino acids, most often Pro and Arg together

The porcine PR-39 is multifunctional, with a potent antibiotic activity, a wound healing function and an antioxidant role [20–22]. There are larger cousins of PR-39 called Bac5

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Abbreviations: cfu, colony forming unit

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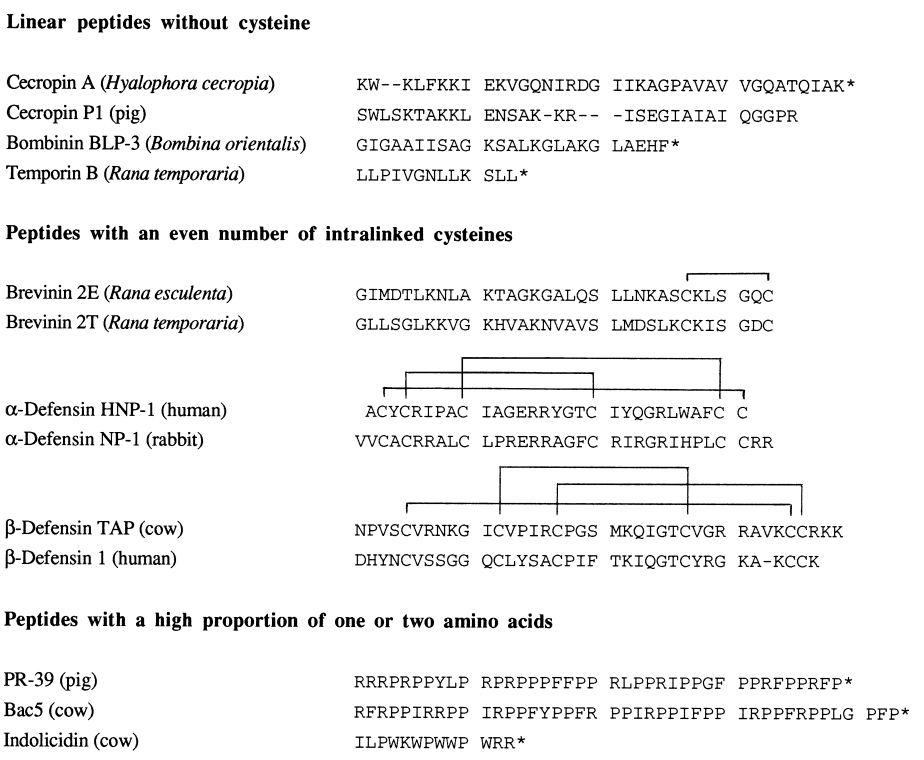

Fig. 1. Sequences of representative peptide antibiotics from the three main superfamilies. The presence of an amidated C-terminus is indicated by the asterisk. So far the linear peptides and the Cys-containing peptides represent the two largest groups, numbering more than 100 sequences each. Only one example of S–S linkage is given for each group of peptides. For four peptides, activities against different bacteria are given in Table 1. For available sequences, see a data base: http://www.bbcm.univ.trieste.it/–tossi/ search.html.

and Bac7 from cow [23]. The latter animal also makes indolicidin, a 13 amino acid peptide containing five Trp residues [24].

3. Peptide precursors

All gene-encoded peptide antibiotics are made as inactive precursors, usually 2–5 times larger than the active effector molecules. It has often been found that different peptides from related animals have proparts with highly conserved sequences. Cathelicidins from mammals are an example of a highly variable peptide family with at least 20 members, all having a strongly conserved propart [25]. Both PR-39, Bac5, Bac7 and indolicidin belong to the family of cathelicidins. Four gene sequences (one human and three from pig) show that the conserved information for the signal and the cathelin proparts are encoded in the first three exons, while the variable effector part is in exon 4 [26–28]. In the defensins, the function of the propart is to direct the mature molecule to the right type of granules, but the role of the cathelin propart is not yet fully understood. In both defensins and cathelicidins, the acidic propart may decrease or prevent the membrane interaction of the basic effector part [29].

In amphibia it has been shown by cDNA sequencing that peptides with different biological activities, either antimicrobial or pharmacological, have similar structures in the propart of their precursor sequences. In different *Rana* species, antimicrobial peptides such as brevinins and esculentins [18], temporins [16] or ranalexin [30], share their propart with peptides from different *Phyllomedusa* species, like dermaseptins [31] or the opioid peptides dermorphins and deltorphins [32].

4. Gene regulation of peptide antibiotics

Thanks to all the genetic know-how accumulated for *Drosophila*, this is without comparison the organism in which the gene control of peptide antibiotics is best understood [6,7]. The promoter regions of inducible peptide antibiotics are often (or always?) regulated by the transcriptional control machinery, NFκB-IκBα, originally discovered by Baltimore's group and now the subject of intense research activity [33]. This machinery seems to be so strongly conserved that *Drosophila*, which offers convenient experimental conditions both with live flies and cell lines, may serve as a model system for mammals.

A significant amount of these regulatory studies has been done in mammalian cell cultures and this applies also to the demonstration that glucocorticoids induce a constitutive synthesis of IκBα [34,35]. However, immune function cannot be reliably studied in cell lines, and consequently genetically modified mice are beginning to take central position in works aimed at understanding the in vivo role of different cytokines, perforin and receptors. Mice are known to have 17 different enteric defensins [36] and one gene homologous to human β-defensin 1 has been analyzed [37]. However, mice do not have peptide antibiotic-containing neutrophils. Despite this fact, it is universally accepted that mice are the best mammalian disease models for humans.

In this review, we intend to focus on vertebrates, and particularly amphibia, that can function as model systems for gathering both in vivo and in vitro data for the natural function of peptide antibiotics. In fact, as discussed below, frogs may offer advantages over murine models.

5. Frogs as model systems

Erspamer's pioneering work showed that frog skins are a rich source of neuropeptides, many identical to those present also in human tissues, albeit in much lower quantities [38]. Zasloff realized that frogs have the same capacity to fight infections [11] as demonstrated 6 years earlier for insects. As the result of his subsequent work, analogues of magainin from *Xenopus laevis* have now passed phase three of clinical trials and may soon be on the market as human drugs for cutaneous infections. However, magainins are unique to *X. laevis* and other species of frogs have been found to have a wide variety of different antimicrobial peptides in their skin secretion, mostly linear, but some with one pair of intralinked cysteines [17]. A particular advantage of the frog is that the skin secretion can be stimulated by a mild electric shock and the excreted peptides are easily washed off, collected and analyzed. Thus, the same animal can be used for repeated experiments, which is not possible with mice. Another advantage of frogs is that biosynthesis of active peptides may involve up to three post-translational modifications: (i) a processing that gives an N-terminal pyroglutamate, (ii) a C-terminal amidation (taking the amide group from a post-ultimate Gly residue), and (iii) a conversion of an encoded L-amino acid to its D-isomer [39]. The latter reaction is a fast biochemical way by which an inactive precursor can be converted in one step to a potent physiological agent.

We are investigating the natural flora of frogs. Relatively few bacterial species are present in a wild animal; four of the most frequent are listed in the top part of Table 1. They are all known to be present in the normal flora of humans. In the case of *Aeromonas hydrophila*, it is probably always part of the normal flora, but only after viral infections (detected or undetected) or in individuals with an impaired immune system can it be diagnosed as a pathogen.

To perform experimental infections, spontaneous and stable antibiotic-resistant mutants were isolated from *A. hydrophila* and *Enterobacter agglomerans*. These two mutants were used for double infecting the mouth of *Rana esculenta*. One set of frogs was kept as control and another was pretreated with a glucocorticoid (GC) cream on the skin [40]. The mouth of the frogs was infected with 2×10^6 cells of each bacterial strain. The progression of the infection was monitored by pipetting 20 μl LB into the frog mouth and quickly withdrawing 5 μl 'saliva' for viable counts. Fig. 2 shows the results of one such double infection. In the wild healthy frog, in which the bacterial levels are normally 0–5 cfu/5 μl, there was a small temporary increase (73 and 26 cfu/5 μl of *A. hydrophila* and *E. agglomerans*, respectively). At day 12, no antibiotic-resistant bacteria were found in the 'saliva' of the control. The GC-treated frog gave a transient infection with *A. hydrophila* (max. 1654 cfu/5 μl at day 6) and a progressive infection with *E. agglomerans* (4400 cfu/5 μl at day 12). Thus, the normal capacity to adjust an excess of a natural component of the flora is lost after GC treatment.

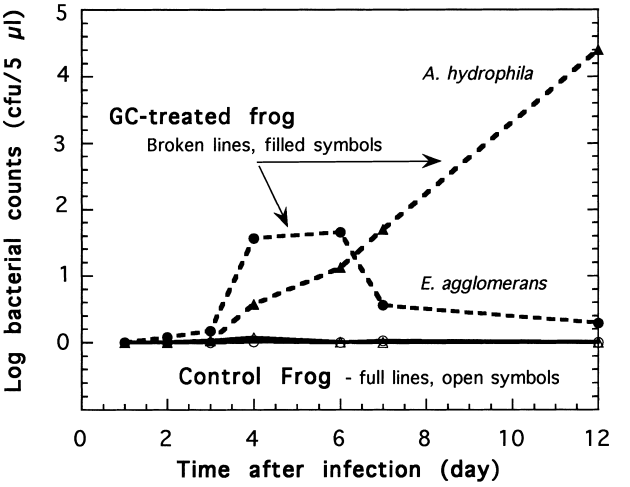

Fig. 2. Effects of glucocorticoid treatment on the bacterial levels in the mouth of *Rana esculenta* frogs, double infected with antibiotic-resistant strains of *Aeromonas hydrophila* (triangles) and *Enterobacter agglomerans* (circles). The skin of the GC-treated frog was given a systemically acting cortisone cream, as reported [40]. Data for known antibacterial activities against the two infecting bacteria are given in Table 1.

We know from recent experiments that the genes for frog antibacterial peptides are down-regulated by GC treatment, while IκBα is clearly up-regulated [40]. So far, all genes for inducible peptide antibiotics in animals have been found to have potential binding sites for NFκB [7,27,37,41,42] and those of mammals also for NF-IL6. This experiment on amphibia implies that frog genes for peptide antibiotics are under control of the dual NFκB-IκBα system. However, only a few frog peptide antibiotic genes have been sequenced and in no case have the promoter regions been discussed.

The peptides and the cDNA of *Bombina orientalis* [14] and

Table 1
Antibacterial activity of frog skin secretions and some purified peptides

Organism	Strain	Skin secretion (cecropin U/mg)			Peptide (LC values, μM)			
		Rana esculenta	*Rana temporaria*	*Bombina orientalis*	Brevinin 2E	Brevinin 2T	BLP-3	Cecropin P1
Enterobacter agglomerans	Bo-1S	30	50	19	0.9	2.1	1.9	0.6
Aeromonas hydrophila	Bo-3N	1.7	1.0	1.5	30.0	30.0	25.7	2.6
Klebsiella pneumoniae	Rt-1	30	50	15	2.2	0.5	7.4	0.5
Acinetobacter junii	Bo-2	110	140	100	0.2	8.5	1.3	0.6
Escherichia coli	D21	270	120	140	0.5	0.5	3.0	0.3
Bacillus megaterium	Bm11	>600	1000	390	0.2	0.2	0.8	5.3
Yersinia pseudotuberculosis	Wild type III	>600	>900	>650	0.1	0.2	0.5	0.5

The first four organisms are isolates from frog mouth or skin, the next two are our common test bacteria. The *Yersinia* strain was included as an example of a human pathogen (kindly provided by Hans Wolf-Watz, Umeå). The sequences of the four peptides are given in Fig. 1. The antibacterial activity of the whole secretion is given as cecropin units/mg. The LC value (μM) is the lowest concentration that inhibits growth in a zone assay. The porcine cecropin P1 was included as a well known reference.

Bombina variegata [13] were investigated in parallel. We have recently completed the DNA sequence of two genes in *B. orientalis*, encoding two copies each of bombinin-like peptides and, in addition, two peptides not identified before [43]. In the upstream regions, binding sites were found for NFκB, which was expected, but also for NF-IL6. Experiments to demonstrate the function of the promoter regions are in progress.

Can Table 1 explain the outcome of the double infection shown in Fig. 2? So far, the answer is yes, because the skin secretion of *R. esculenta* is more than 15 times more effective in killing *E. agglomerans* than it is in killing *A. hydrophila* (Table 1). The latter is one of the bacteria in the normal flora that is most resistant to the peptides tested.

6. When does nature worry about the defense budget of animalcules?

When comparing innate and acquired immunity it is relevant to ask how much DNA is needed in each case and how much energy is consumed. If we consider that the same amounts of ATP and GTP are needed for each peptide bond formed, we come to a 10-fold difference in energy requirement for making one molecule with 100 amino acid residues compared to one molecule of IgG with around 1000 residues. If we then take into account that the specificity of the peptide antibiotics is relatively wide (perhaps 20–30 different peptides can protect an animal), while the high specificity of immunoglobulins is quite expensive (estimates run at around 10^6 different antibodies in a mouse), the amount of chemical energy used by classical immunology is orders of magnitude larger than that used by innate immunity. Thus, as long as self-destruction is avoided, a low-specificity immune mechanism is much more cost-effective than one with a maximum of specificity.

Looking at the DNA, a rough calculation would show that in order to make an IgG molecule, repertoires of gene segments are needed which make the completed molecule to require 13.4 Mb [44]. A family of 20 peptide antibiotics, each with four assumed processing enzymes, may only need some 130 kb [45] which is in the order of 1%. It is hard to escape the notion that this has influenced the evolution of animals. If the rate of protein synthesis is more or less a temperature-dependent constant, then the biomass of a species (number of individuals and body size) may vary chiefly with evolution (including microbe interaction). However, body size is critical, because small animals like insects cannot allow space for repertoires and large mammals with a slow rate of reproduction have had to provide the extra DNA needed for classical immunity. In this respect, the frogs are a mystery (and perhaps a border case), because they have lymphocytes and capacity for a repertoire (shown to produce antibodies for DNP-haptens).

An infection can end in the following three ways, which are fundamentally different and may depend on the rate of reproduction: (1) the invading microbe is completely eliminated, an event that is both possible and desirable; (2) a compromise between the host and the parasitic organism is reached, a process that is a biological necessity; (3) the infecting organism is added to the natural flora, with the physiological regulation that this would require. For bacteria and alternatives (1) and (3) there are strong indications that gene-encoded peptide antibiotics are the effector molecules. Obligate parasites like viruses require alternative (2), the compromise, and that is what most immunologists so far have been preoccupied with.

Only the future will show to what extent each of these three alternatives reflects what goes on in nature in wild animals and also what was taking place in our ancestors 20 000 years ago. Moreover, recent estimates indicate that we may know only about 1% of the microbial world [46]. If the immune system is supposed to protect us from these microbes and if most of them are still unknown to us, we could in fact have an incomplete understanding of what goes on. Also, one or another misinterpretation could stem from some unphysiological experiments in the past.

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